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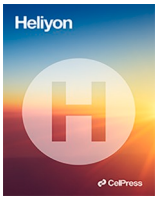

Research article

Network pharmacology and experimental validation to explore the mechanism of Changji'an formula against irritable bowel syndrome with predominant diarrhea

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ABSTRACT

Changji'an Formula (CJAF) is a Chinese herbal compound, which is effective against irritable bowel syndrome with predominant diarrhea (IBS-D) in clinic. However, the molecular mechanism has not been well defined. In the current study, the potential targets and signaling pathways of CJAF against IBS-D were predicted using network pharmacology analysis. The pharmacological mechanisms of CJAF against IBS-D and the potential mechanism were validated by using an IBS-D mouse model induced by enema with trinitrobenzene-sulfonic acid (TNBS) plus with restraint stress and further intervened with CJAF. A total of 232 active compounds of CJAF were obtained, a total of 397 potential targets for the active ingredients were retrieved and a total of 219 common targets were obtained as the potential targets of CJAF against IBS-D. GO and KEGG enrichment analyses showed that multiple targets were enriched and could be experimentally validated in a mouse model of IBS-D. The mechanisms were mainly converged on the immune and inflammatory pathways, especially the NF-κB, TNF and IL-17 signaling pathway, which were closely involved in the treatment of CJAF against IBS-D. Animal experiment showed that CJAF alleviated visceral hypersensitivity and diarrhea symptom of IBS-D. CJAF also restored the histological and ultrastructure damage of IBS-D. The result of Western blot showed that CJAF upregulated colonic tight junction proteins of ZO-1, Occludin and Claudin-1. Further results demonstrated that CJAF inhibited the protein expression of NF-κB/NLRP3 inflammasome

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pathway targets and downregulated proinflammatory mediators of IL-1β, IL-18, TNF-α. In conclusion, CJAF could effectively reduce inflammatory response and alleviate visceral hypersensitivity as well as diarrhea symptom of IBS-D by inhibiting the NF-κB/NLRP3 signaling pathway. This study not only reveals the mechanism of CJAF against IBS-D, but also provides a novel therapeutic strategy for IBS-D.

1. Introduction

Irritable bowel syndrome (IBS) is a common gastroenterological disorder in clinic, with a global prevalence of 7%–21 % [1]. According to the Rome IV criteria, IBS can be classified into 4 main subtypes, of which, IBS with predominant diarrhea (IBS-D) maybe the most troublesome subtype, which could result in a worse disease-specific quality of life [2,3]. Moreover, the pathogenetic mechanism of IBS-D is complex and not fully understood and the therapeutic strategies are not satisfactory [4]. Therefore, new therapeutic strategies are desperately needed for a better care and handling of IBS-D.

Recently, the interest in traditional Chinese medicine (TCM) has increased all over the world, owing to its distinctive properties. The strong points of TCM attributes to its effect on multiple molecular targets and signaling pathways associated with the same disease as opposed to a single molecule, whilst causing few side effects. According to the theory of TCM, IBS-D can be classified into the categories of “abdominal pain”, “spleen deficiency” and “diarrhea” and clinically, the syndrome of liver depression and spleen deficiency is the most common type [5]. Changji’an Formula (CJAF) is the combination and transformation of two famous classical prescription: Sijunzi decoction and Tong-Xie-Yao-Fang, which functions as soothing the liver and strengthening the spleen, relieving pain and preventing diarrhea, and have been widely used to treat IBS-like symptom diseases or IBS-D in clinic [6–10]. Moreover, Our previous studies demonstrated that CJAF could attenuate typical symptoms of IBS-D by regulating immune function and improving intestinal permeability of IBS-D rats [11,12]. However, the mechanism of CJAF against IBS-D still needs to be clarified.

Network pharmacology research is built on the foundation of integrating network biology and polypharmacology [13], which focuses on multiple nodes in a targeted biological network system instead of a single molecule, resulting in better therapeutic effects and few side effects [13,14]. This is in keeping with the holistic theory of TCM, which implements therapy according to syndrome differentiation from a systemic perspective. Network pharmacology have been increasingly employed in the investigation of TCM to predict the main pharmacological component, potential target groups and their mechanisms of action [15]. Therefore, the aim of this study is to explore the mechanism of CJAF against IBS-D by network pharmacology and further to validate it by IBS-D mouse model.

2. Materials and methods

2.1. Screening for active ingredients of CJAF

CJAF consists of thirteen medicinal herbs, including Baizhu (BZ), Baishao (BS), Huangqi (HQ), Fuling (FL), Zhiqiao (ZQ), Shiliupi (SLP), Yanhusuo (YHS), Huanglian (HL), Wumei (WM), Fangfeng (FF), Chenpi (CP), Gancao (GC) and Chaihu (CH) (Supplementary Table 1). The active ingredients of the herbs in CJAF were obtained from TCMSP database (https://tcmspw.com/tcmspsearch.php) and selected using the in silico integrative ADME model. Those ingredients were selected only if oral bioavailability (OB) $\geq$ 30 and drug likeness (DL) $\geq$ 0.18 [16–18].

2.2. Screening of potential targets for active ingredients and IBS-D

TCMSP (http://tcmspw.com/) [18] and PharmMapper (http://www.lilab-ecust.cn/pharmmapper/) [19] databases with the “*Homo sapiens*” setting, were used to screen the potential targets of those active components in CJAF. Then, duplicates were removed. Potential targets associated with IBS-D were excavated from the GeneCards (http://www.genecards.org), Therapeutic Target Database (TTD, http://systemsdock.unit.osit.jp/iddp/home/inde), DrugBank (http://www.drugbank.ca/) and DisGeNET (http://www.disgenet.org/) databases [16,17]. Finally, all the predicted targets for compounds and diseases were transformed into corresponding gene symbols using UniProt database (https://www.uniprot.org/) [20].

2.3. Network construction

To get a comprehensive understanding of the molecular mechanisms of CJAF in the treatment of IBS-D, we constructed a network. The common targets of the active ingredients and IBS-D were obtained using Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/). Then, the protein-protein interaction (PPI) network of the common targets was established by using STRING database (https://string-db.org/) and further visualized by using Cytoscape software [16–18]. Finally, crucial targets of the active components in CJAF against IBS-D were obtained based on the degree values in the Cytoscape settings.

2.4. Enrichment analysis

The Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were

conducted by using Omicshare Online tools (https://www.omicshare.com/tools) [20]. GO (https://geneontology.org/) is a commonly adopted ontology in bioinformatics scope [17]. The threshold was set as *P* adjust value < 0.05 and the top 25 of GO terms enrichment were present. KEGG (https://www.kegg.jp/) is a practical database to interpret the biological function of candidate targets [17]. *P* adjust value < 0.05 was regarded as the threshold and the top 25 enriched KEGG pathways were displayed.

2.5. Drugs and reagents

CJAF consists of 13 Chinese herbal medicines. These herbal medicines were obtained from Chinese herbal medicine factory of Guangdong Pharmaceutical Company (Guangzhou, China). Other detailed information about the medicinal materials was described in our previous study [21], with the English version of which was supplemented in Supplementary Table 1. Rifaximin (Alanno, Italy; lot no. 22953) was selected as a positive drug, which is an FDA-approved, nonabsorbable, oral antibiotic for IBS-D [22]. Rifaximin was also shown to be effective against IBS-D rat model by restoring intestinal microecology and preventing intestinal inflammation as well as visceral hypersensitivity induced by stress [23]. Trinitro-benzene-sulfonic acid (TNBS) were purchased from Sigma-Aldrich (Lot NO. SLCD2161, St. Louis, MO, USA). Stripping buffer were supplied by CW-BIO (Jiangsu province, China. Lot No. 01427/12322).

2.6. Preparation of CJAF extract

CJAF was extracted by boiling the herb medicinal materials in appropriate amount of water for 30 min and repeated once. The water extract solution was merged together, centrifuged at 3000 *g* for 10min, and further filtered by filter paper to remove the impurities. Then the water extract solution was condensed using a rotatory evaporator at 60 °C until water extract solution became thick paste. Then the thick paste was frozen at −80 °C before transferred into a vacuum freeze drier (Biosafer-18D, Nanjing Saifei Biotechnology Co., LTD, Nanjing, China) for 48–72 h. The freeze-dried powder of CJAF were converted as crude drug (gram) per gram of freeze-dried powder. Analysis of the main chemical compounds from freeze-dried powder of CJAF were described in our previous studies [21,24].

2.7. Animals and experimental design

This research was approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine (certificate no. 20220510014). Animals were handled in accordance with the regulations of Guangzhou University of Chinese Medicine and the ARRIVE guidelines. All mice were free access to feed and water with a 12 h light-dark cycle (25 ± 1 °C). Eight-week-old male mice weighing 20–25 g was used in this study.

IBS-D was induced by a single dosage of rectal administration of 100 μL 50 mg/kg TNBS (dissolved in 50 % ethanol) combined with restraint stress 2 h per day for 7 consecutive days, as described in detail previously [25]. Mice in normal control group received distilled water instead of TNBS in enema and did not receive restraint stress. After IBS-D model establishment, mice were left for recovering for 7 days and then they were randomly divided into normal control group(NC, n = 7), model control group (MC, n = 7), rifaximin group(100 mg/kg, n = 7), high dosage of Changji'an Formula group (CJAF-H, 12.91 g(freeze-dried powder)/kg(body weight), n = 7), moderate dosage of Changji'an Formula group (CJAF-M, 6.45 g/kg(freeze-dried powder), clinical equivalent dose for humans, n = 7), low dosage of Changji'an Formula group (CJAF-L, 3.23 g/kg(freeze-dried powder), n = 7). NC and MC groups received distilled water. Rifaximin and freeze-dried powder of CJAF were all dissolved in pure water, respectively and administered through gavage for 7 days once per day. The dosage of rifaximin (100 mg/kg) was according to a previous study [26]. The dosage of CJAF was converted from clinical equivalent dose for humans and further converted by the yield rate of freeze-dried powder.

After administration, mice were anesthetized by pentobarbital sodium (50 mg/kg) via i.p. before the collection of blood and colonic tissue samples. Serum was obtained by centrifuging the blood sample at 1000 *g* at 4 °C for 15 min. Serum and colon tissue samples were stored at −80 °C until further use.

2.8. Assessment of IBS-D symptoms

2.8.1. Diarrhea score, fecal water content

These experiments were carried out at the end of administration. The severity of diarrhea was scored as previously reported [27]. In brief, mice were housed in a metabolic cage for 1 h with water accessible freely. The fecal pellets expelled during this 1 h period of time was collected and counted. The diarrhea score was evaluated: 1 = normal and dry pellet; 2 = normal and wetter pellet; 3 = wet and less-shaped pellet; 4 = complete diarrhea. And then fecal pellets were dried until reached constant weight. The reduced weight is water.

2.8.2. Water avoidance stress (WAS) stimulated fecal pellet output

The procedure was conducted after drug administration and was described elsewhere [28], with minor adjustments. In brief, mice were placed in a platform (L × W × H = 6 cm × 6 cm × 15 cm) for 1 h in a cuboid container (L × W × H = 26 cm × 26 cm × 35 cm) at room temperature. The cuboid container was filled with water to the height of 1 cm below the platform. After 1 h of WAS, the number of fecal pellets output by each mouse were counted and the total number were averaged for each group.

2.8.3. Visceral sensitivity

The visceral sensitivity of mice was evaluated by abdominal withdrawal reflex (AWR) under graded pressure of colorectal distension (CRD). To produce visceral pain, different volumes of water was filled into a 6F catheter, which was inserted into the colon 2–3 cm from anus [29]. Semi-quantitative AWR score (0–4) was used to evaluate AWR score at different grades (distension volume was 0.15, 0.30, 0.45, 0.60 mL) of CRD. The higher the score, the more sensitive is the viscera. To ensure accuracy, all pressure was repeated three times and averaged and the AWR scores were evaluated by two independent operators blinded to animal group.

2.9. Histopathological examination

Colon tissues of each mouse were fixed in 4 % paraformaldehyde and further embedded in paraffin (Servicebio, Wuhan, China) after sample collection. Then they were cut into 5 μm sections with a microtome (Leica, Wetzlar, Germany). And then the sections were treated with xylene I, xylene II and with mixture of ethanol and xylene (1:1), then they were dewaxed and dehydrated. The sections were then supplemented with hematoxylin and eosin to demonstrate epithelial structure and infiltration of inflammatory cells. Two pathologists blinded to the animal grouping analyzed the slices and the morphological structure of mice colon tissues was scored as previously described [30].

2.10. Transmission electron microscopy (TEM)

Colon tissues of mice were processed for TEM as previously reported with minor adjustment [31]. In brief, fresh colon tissue were cut into small pieces (<1 mm × 1 mm × 1 mm), and fixed in 2.5 % buffered glutaraldehyde for 2–4 h at 4 °C. Then washed with phosphate buffer (PB, PH 7.4) for three times, 15 min each. And the tissues were further fixed in mixed solution of 1 % osmic acid and 0.1 mol/L PB (PH 7.4) for 2 h at 25 °C, followed by being washed with 0.1 mol/L PB (PH 7.4) for 3 times, 15 min each. Then colon tissues were dehydrated in 50 %–70 %-80 %-90 %-95 %-100 %-100 % ethyl alcohol-100 % acetone-100 % acetone, each step lasted for 15 min. Then penetrated in acetone-Epon812 (1:1) for 2–4 h and further penetrated in acetone-Epon812 (2:1) overnight and embedded in Epon812 for 5–8 h. Then filled the embedded plate with Epon812 and inserted samples into the embedded plate in an oven at 37 °C overnight. After polymerization in 60 °C for 48 h, ultrathin slices were cut (60–80 nm). Then stain the slices with 2 % uranium acetate saturated alcohol solution and with lead citrate, 15 min for each step. The sections were observed using an HT7800/HT7700 TEM (Hitachi, Tokyo, Japan).

2.11. ELISA

The serum was thawed at 4 °C and diluted in the ration of 1:10–1:20 before test. Diamine oxidase (DAO), D-lactate (D-LA), IL-1β, IL-18, TNF-α, and myeloperoxidase (MPO) of serum were quantified by using commercially accessible ELISA kits by following the manufactures' instructions. Optical density was determined at 450 nm wavelength by using a microplate reader.

2.12. Western blotting

Mice colonic proteins were extracted using RIPA lysis buffer (Beyotime, China, P0013B) added with 1 % phenylmethanesulfonyl fluoride (PMSF, Beyotime, China, ST505), 1 % phosphatase inhibitors A (Beyotime, China, P1081) and 1 % phosphatase inhibitors B (Beyotime, China, P1086). After proyein loading, protein blots were transferred onto 0.45 μm bore diameter polyvinylidene difluoride (PVDF) membranes at 250 mA for 60 min. After blocking with 5 % albumin bovine V (Solarbio, China, Cat#A8020) at 25 °C for 1 h, the membranes were incubated at 4 °C with primary antibodies overnight against IκBα (1:1000, ab32518, Abcam, Cambridge, MA, USA), p-IκBα(Ser32) (1:1000, #2859, Cell Signaling Technology, Danvers, MA,USA), NF-κB(p65) (1:1000, ab32536, Abcam, Cambridge, MA, USA), phospho-NF-κB p65 (Ser536) (1:1000, #3033, Cell Signaling Technology, Danvers, MA,USA), NLRP3 (1:1000, #15101, Cell Signaling Technology, Danvers, MA,USA), ASC (1:1000, #67824, Cell Signaling Technology, Danvers, MA,USA), pro-caspase-1/caspase-1(1:1000, 22915-1-AP, Proteintech, Wuhan, China), IL-1β(1:1000, Proteintech, 26048-1-AP, Wuhan, China), IL-18 (1:500, #DF6252, Affinity Biosciences, Cincinnati, OH, USA), TNF-α (1:500, #AF7014, Affinity Biosciences, China), Claudin-1 (1:1000, 13050-1-AP, Proteintech, Wuhan, China), Occludin (1:2000, 27260-1-AP, Proteintech, Wuhan, China), ZO-1 (1:1000, 21773-1-AP, Proteintech, Wuhan, China) and GAPDH (1:1000, ab181602, Abcam, Cambridge, MA, USA). Then the membranes were washed with 1 × TBST for 3 times, 5 min for each time and incubated with goat against rabbit IgG H&L (HRP) (ab205718, abcam, Cambridge, MA, USA) for 1 h at 25 °C. The protein blots were visualized and quantified in standard routine operation. GAPDH is used as an internal reference and all the results were normalized to NC group. Protein bands were indicated by PageRuler Prestained Protein Ladder (Thermo Scientific, #26616) and Precision Plus Protein™ Dual Color Standards (biorad, #1610374).

2.13. Statistical analysis

The statistical analyses were conducted using the IBM SPSS software v25.0 (IBM Corp., Armonk, NY, USA). Shapiro-Wilk normality test was used to test the normality of the data. Parametric tests were performed to analyze the normal distributed data. Data were expressed as the mean ± SD and were analyzed by one-way ANOVA followed by LSD *t*-test for homogeneous variance or Dunnett's T3 for inhomogeneous variance. The differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Screening for the active ingredients of CJAF

A total of 232 active ingredients were gained in terms of good ADME properties, including 7 active chemical compounds of BZ, 13 active chemical compounds of BS, 20 active chemical compounds of HQ, 15 active chemical compounds of FL, 5 active chemical compounds of ZQ, 7 active chemical compounds of SLP, 49 active chemical compounds of YHS, 14 active chemical compounds of HL, 8 active chemical compounds of WM, 18 active chemical compounds of FF, 5 active chemical compounds of CP, 92 active chemical compounds of GC, and 17 active ingredients of CH (Fig. 1).

3.2. Potential targets for active ingredients and IBS-D

397 potential targets for the active ingredients were retrieved by screening the databases of PharmMapper and TCMSP (Fig. 2A). 1299 potential targets for IBS-D were obtained by screening the GeneCards, TTD, DrugBank and DisGeNET databases (Fig. 2A). All these potential targets were identified using the UniProt database. A total of 219 common targets were obtained as the potential targets of CJAF against IBS-D through Venn diagram (Fig. 2A).

3.3. Network construction

The PPI network of the 219 common targets were established using the String database and further visualized with the Cytoscape software platform (Fig. 2B). Then, the topological property of the PPI network was analyzed using the Cytoscape software. A total of 219 nodes and 5576 edges with DC value of 43 were observed in the PPI network (Fig. 2C). Afterwards, hub nodes were identified according to DC values. A total of 111 and 56 hub nodes were identified with DC $\geq$ 43 and 76, respectively. Finally, these 56 crucial targets were identified as the candidate targets of CJAF for the treatment of IBS-D, which included TNF, IL6, ALB, AKT1, TP53, JUN, IL1B, VEGFA, STST3, CASP3, EGFR, MMP9, SRC, MAPK3, MYC, CXCL8, PTGS2, CCL2 and ESR1, etc (Fig. 2D).

3.4. GO and KEGG

The potential 219 common targets were further used for GO analysis and KEGG pathway enrichment analysis to reveal the potential mechanism of CJAF for IBS-D. GO annotation revealed that the common targets were abundantly enriched in biological processes (BP), cell component (CC) and molecular function (MF) (Fig. 3A). The top 25 terms of BP, CC, and MF are depicted in Fig. 3B–D.

According to cell component (CC) analysis, the targets were majorly associated with membrane raft (GO: 0045121), membrane microdomain (GO: 0098857), membrane region (GO: 0098589), plasma membrane part (GO: 0044459), extracellular space (GO: 0005615), etc (Fig. 3C). Based on the molecular function (MF) analysis, the targets were mainly associated with receptor binding (GO:0005102), identical protein binding (GO: 0042802), enzyme binding (GO: 0019899), signal transducer activity (GO: 0004871), cytokine receptor binding (GO: 0005126), protein kinase activity (GO: 0004672), etc (Fig. 3D).

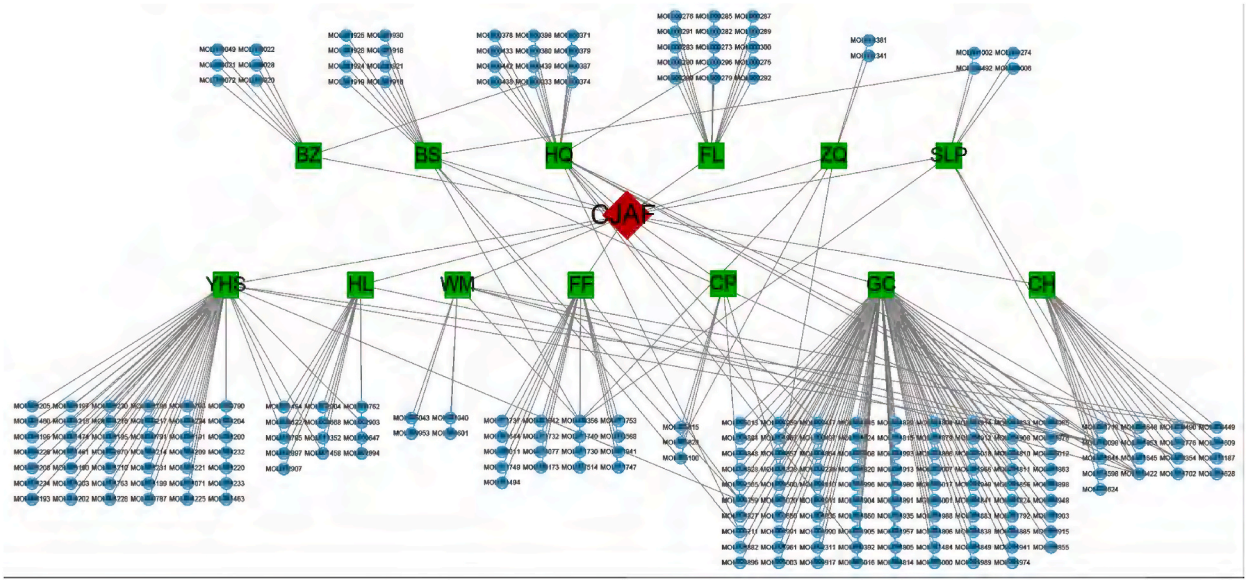

Fig. 1. The medicinal herbs and active ingredients of CJAF. CJAF consists of thirteen medicinal herbs. A total of 232 active ingredients were obtained. 232 active ingredients were accached in supplementary material 1.

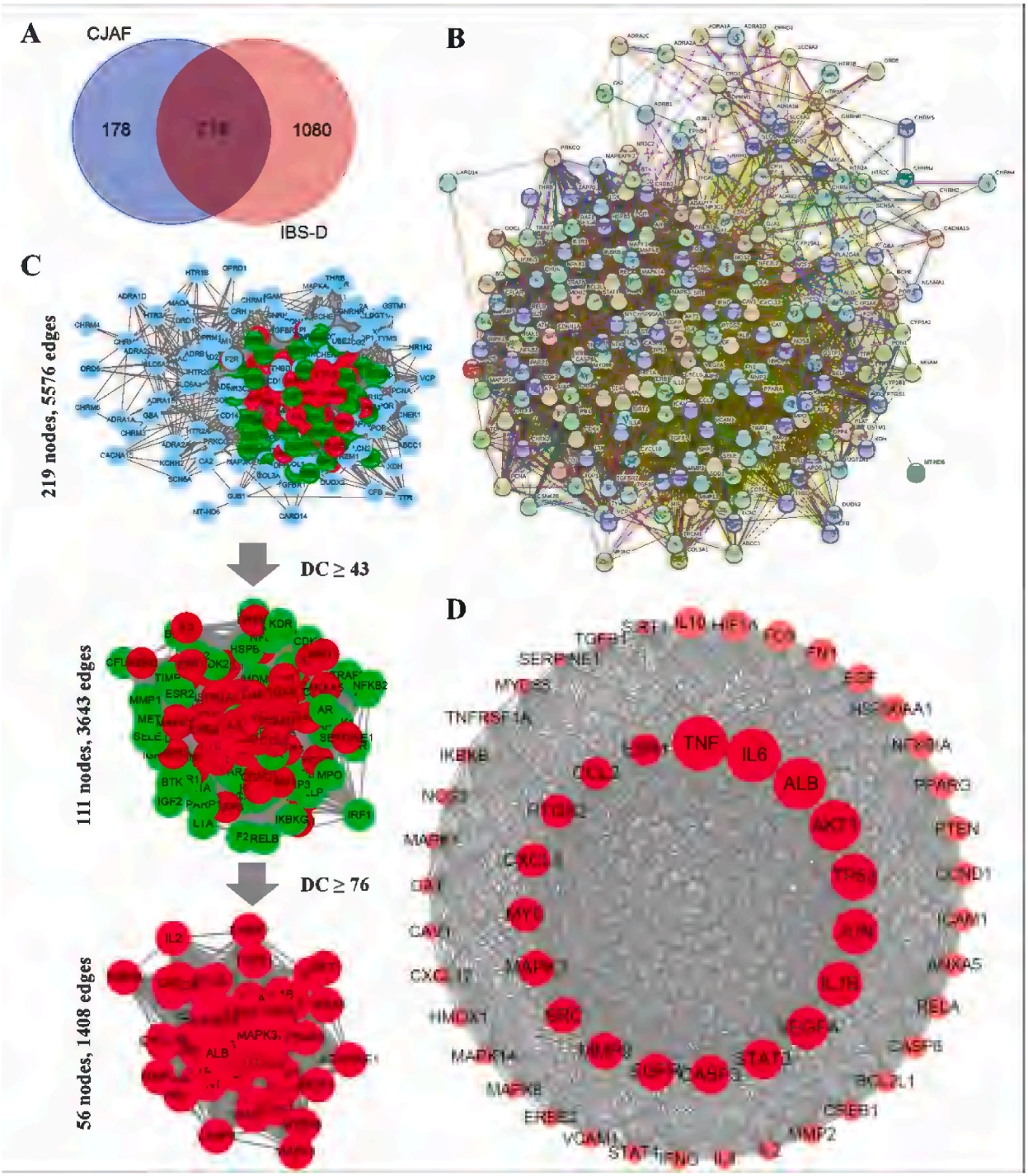

Fig. 2. Potential targets of CJAF against IBS-D. (A) Venn diagram summarizing the 219 common targets for CJAF against IBS-D. (B) The PPI network of the 219 common targets. (C) The process of topological screening for the PPI network of the 219 common targets. A total of 111 and 56 hub nodes were identified when the thresholds were set at DC $\geq$ 43 and 76, respectively. (D) These 56 crucial targets were further analyzed and identified as the candidate targets of CJAF for the treatment of IBS-D. 219 common targets were attached in supplementary material 2.

Additionally, KEGG enrichment analysis demonstrated that the common targets were abundantly enriched in pathways related to metabolism, genetic information processing, environmental information processing, etc. (Fig. 4A). The top 25 KEGG pathway enrichments were showed in Fig. 4B, which include NF-κB signaling pathway, AGE-RAGE signaling pathway in diabetic complications, TNF signaling pathway, pathways in caner, hepatitis B, IL-17 signaling pathway, fluid shear stress and atherosclerosis, osteoclast

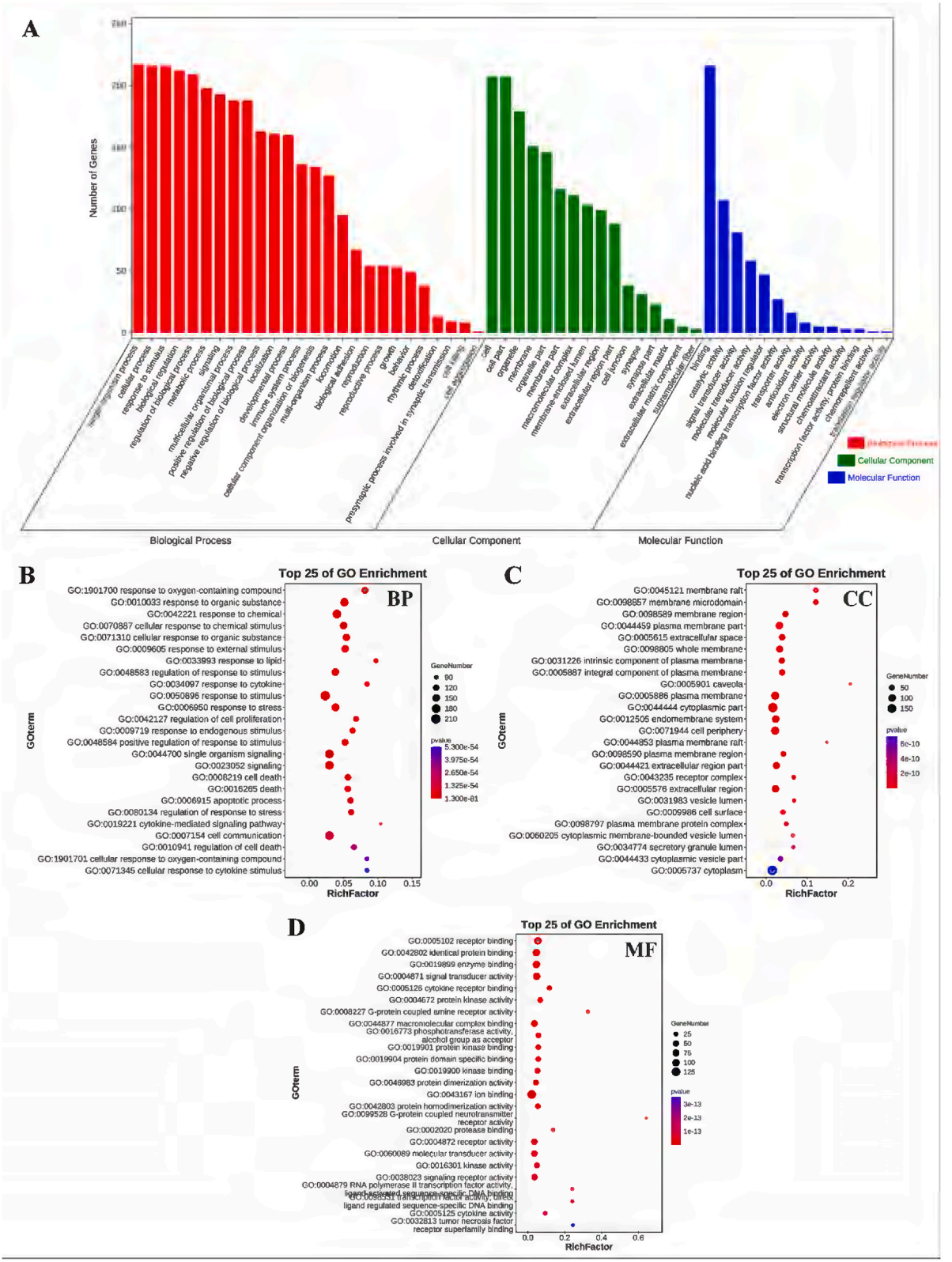

Fig. 3. GO enrichment analysis. (A) Principle genes involved in the biological process (BP), cell component (CC), and molecular function (MF) terms. (B) BP enrichment analysis of the 219 common targets. (C) CC enrichment analysis of the 219 common targets. (D)MF enrichment analysis of the 219 common targets. Only terms with $P < 0.05$ were selected for further analysis. The top 25 enriched GO terms are mapped.

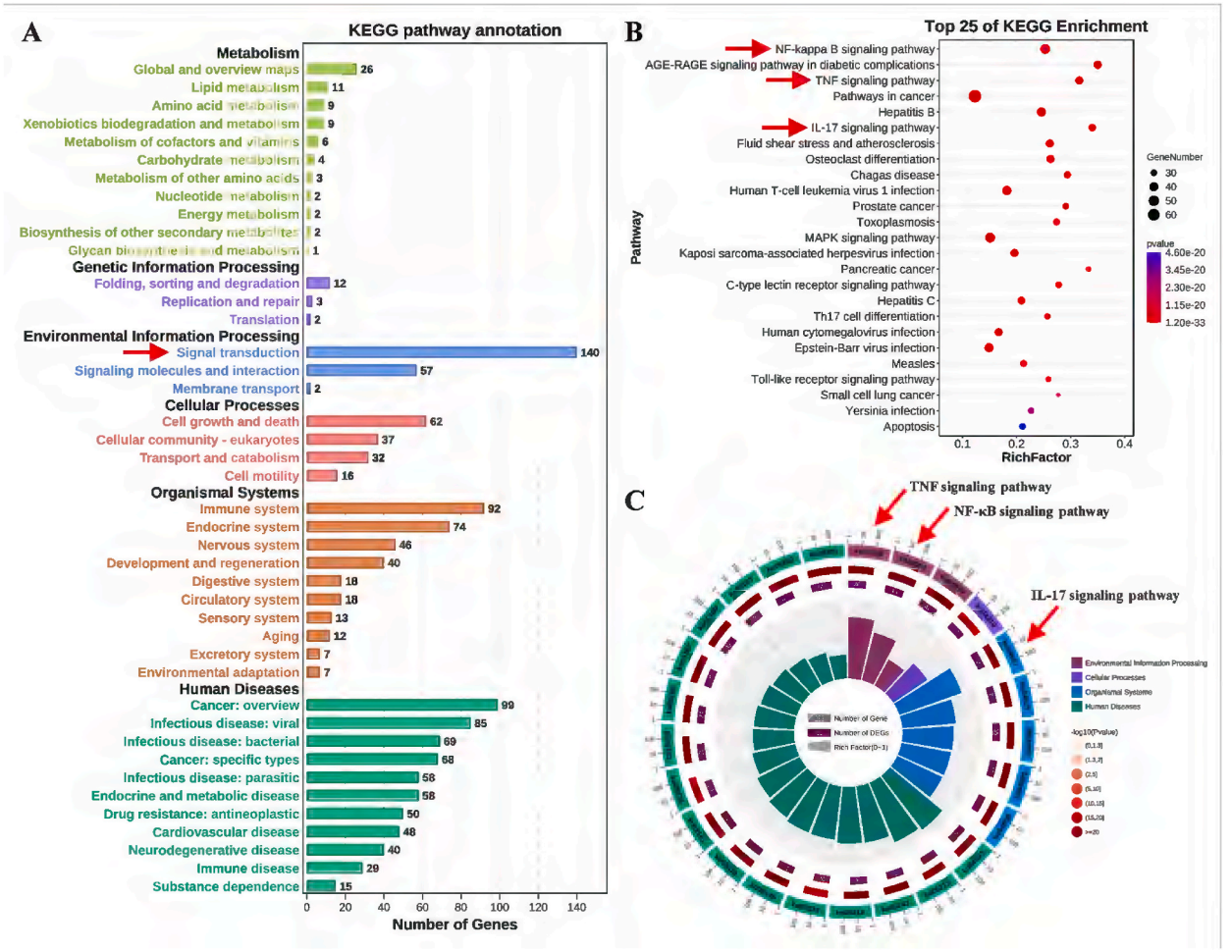

Fig. 4. KEGG enrichment analysis of the common targets of CJAF for the treatment of IBS-D. (A) Genes involved in the KEGG annotation. (B) KEGG enrichment of the 219 targets. Only terms with $P < 0.05$ were selected for analysis. The top 25 enriched KEGG pathways are displayed. (C) The top 25 enriched KEGG pathways.

differentiation, Chagas disease and human T-cell leukemia virus 1 infection, etc. The rich factors of the enriched signaling pathways were displayed in Fig. 4C. And 45, 36 and 33 targets were involved in the NF-κB, TNF and IL-17 signaling pathway, respectively (Fig. 4C).

Schematic drawing to illustrate the NF-κB, TNF and IL-17 signaling pathway involving CJAF against IBS-D was displayed in Fig. 5 A-C. These findings demonstrated that the immune and inflammatory pathways were the predominant molecular mechanisms. Abundant studies have showed that these three signaling pathways are crucial intracellular signal transduction pathways that closely regulate various inflammation-associated diseases. Hence, the potential inflammatory mechanisms related to the NF-κB signaling pathway were further investigated by animal experiment.

3.5. CJAF alleviated main symptoms of IBS-D

To validate the predicted molecular mechanisms from network pharmacology analysis that CJAF could effectively treat IBS-D by inhibiting inflammatory response via the NF-κB signaling pathway, a murine IBS-D model was established and was intragastric administrated with CJAF for 7days. And then macroscopic pathological index of mice, including abdominal pain, and diarrhea score was measured. The micropathological index, including histological examination, ultrastructure of colon tissue was also observed. Moreover, serum levels of proinflammatory cytokines and protein expression of NF-κB/NLRP3 pathway targets were evaluated.

The results showed that the diarrhea score and fecal water content increased significantly in IBS-D group as compared to that of NC group mice ($P < 0.01$, Fig. 6A–B). After the treatment of rifaximin and CJAF, the diarrhea score was decreased ($P_{\text{rifaximin}} < 0.01$, $P_{\text{CJAF-H, CJAF-M}} < 0.05$) and fecal water content was reduced ($P_{\text{rifaximin, CJAF-H,CJAF-M}} < 0.01$, $P_{\text{CJAF-L}} < 0.05$). To characterize the effect of CJAF on the defecation pattern of IBS-D model mice, fecal pellet output under 1 h of WAS was recorded (Fig. 6C). Compared with NC, fecal pellet output increased significantly in MC ($P < 0.01$). After rifaximin, high and moderate dosage of CJAF treatment, fecal pellet output

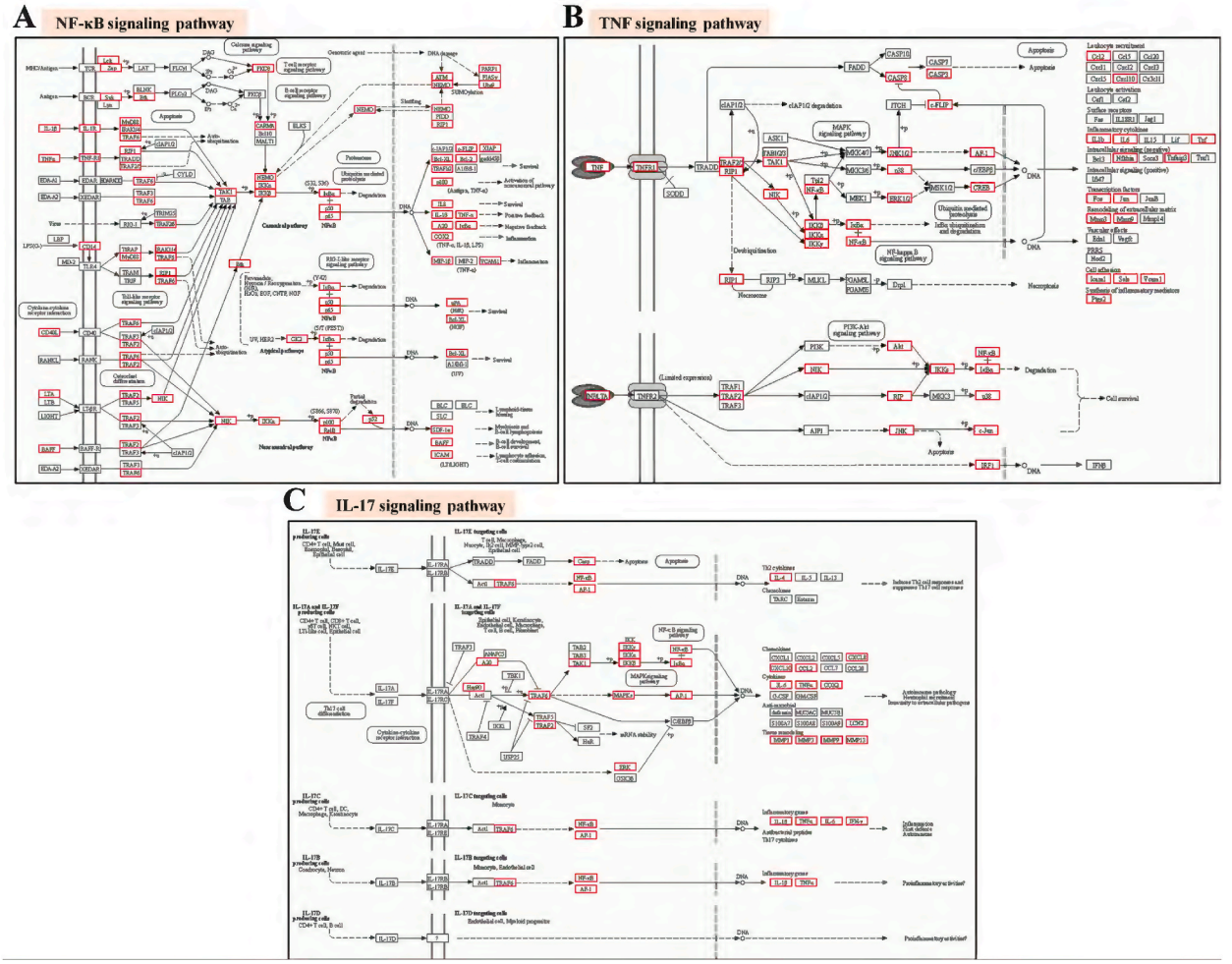

Fig. 5. Principal signaling pathway involved in the treatment of IBS-D by CJAF. Schematic drawing to illustrate (A) the NF-κB, (B) the TNF and (C) the IL-17 signaling pathway involving CJAF for the treatment of IBS-D. The key targets involved in this process are colored in red.

decreased significantly ($P_{\text{rifaximin, CJAF-H}} < 0.01$, $P_{\text{CJAF-M}} < 0.05$). However, CJAF-L did not show alleviation effect on the defecation frequency of IBS-D.

Visceral hypersensitivity is recognized as a principal role in the pathogenesis of IBS-D, characterized by decreased pain thresholds [32,33]. Therefore, we tested the effect of CJAF on the visceral sensitivity of IBS-D by evaluating the AWR score under CRD. As shown in Fig. 6D, MC group mice showed significant higher AWR scores than NC mice at 0.15, 0.30, 0.45, 0.60 mL distension volumes of pressure. Rifaximin, CJAF-H and CJAF-M treatment significantly alleviated pain response of mice at 0.30, and 0.45 mL distension volumes of pressure, compared with that of MC group ($P_{\text{rifaximin, CJAF-M}} < 0.05$, $P_{\text{CJAF-H}} < 0.01$). However, CJAF-L failed to ameliorate visceral hypersensitivity at all distension volumes of pressure compared to that of MC group.

3.6. CJAF ameliorated mice colonic histological damage from IBS-D

The histological structure of mice colon tissue was demonstrated in Fig. 7A. NC group showed undamaged intestinal mucosal layer, ample goblet cells and intestinal glands, normal morphology of the muscle layer and no infiltration of inflammatory cells. In contrast, colonic tissue of IBS-D mice showed broken tissue structure (shown by the blue arrow), damaged mucosal glands and edema (shown by the black arrow), and infiltration of inflammatory cells (shown by the yellow arrow), resulting in a high score of histological damage ($P < 0.01$, Fig. 7B). After treatment of rifaximin and CJAF, the damaged tissue structure was restored, the distribution density of mucosal glands increased, the edema was alleviated and the inflammatory cell infiltration was inhibited in different degrees. Therefore, CJAF promoted histological recovery from IBS-D.

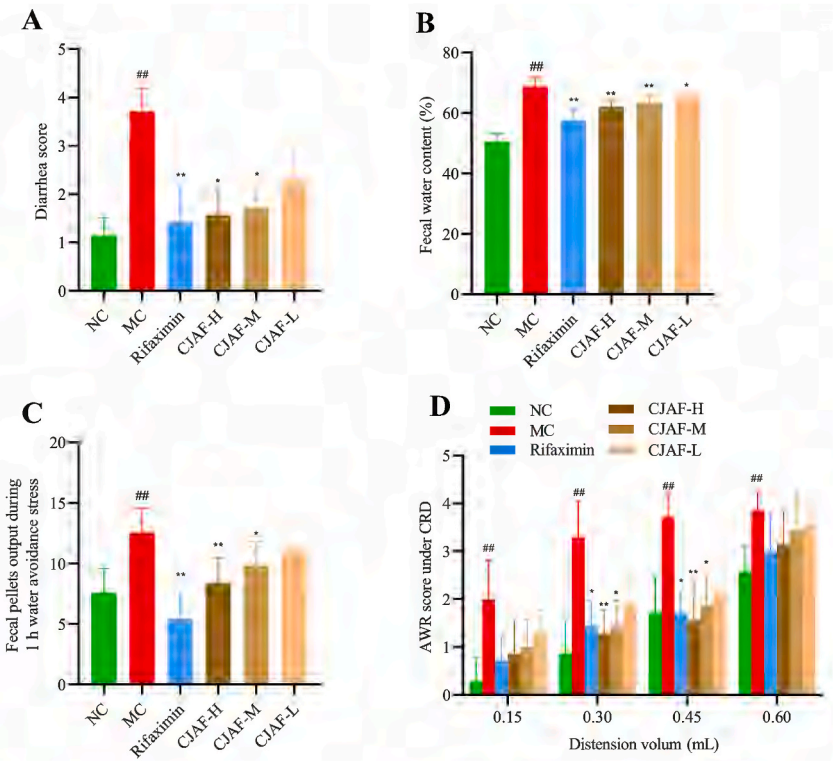

Fig. 6. CJAF alleviated the main symptoms of IBS-D. (A) diarrhea score, (B) fecal water content, (C) fecal pellet output, (D) AWR score under CRD. Results are presented as mean ± SD, n = 7. ($^{\#\#}P < 0.01$ vs. NC; $^{**}P < 0.01$, $^{*}P < 0.05$ vs. MC).

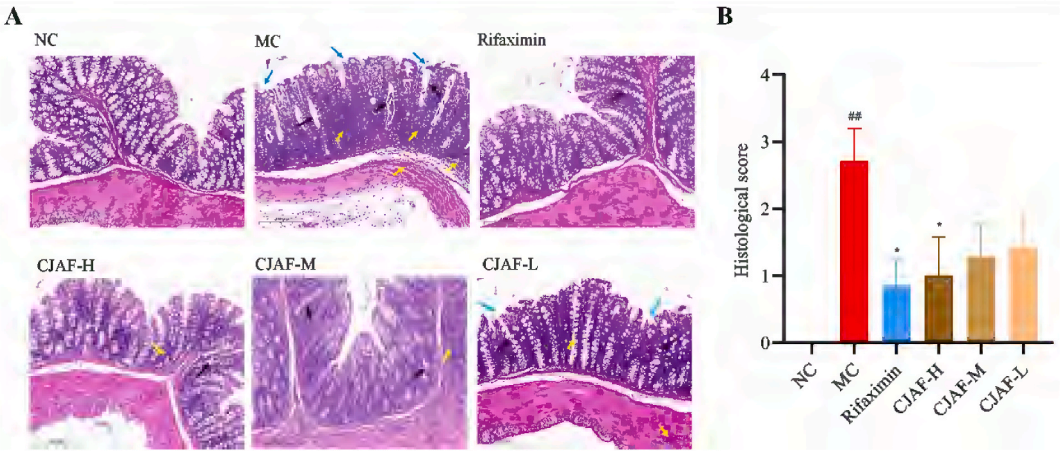

Fig. 7. CJAF promoted histological recovery in IBS-D. (A) Changes in colonic tissue structure after treatment of CJAF: blue arrows indicate defect of tissue structure, black arrows indicate abnormality of mucosal glands and edema, and yellow arrows indicate infiltration of inflammatory cell (scale bars 200 μm). (B) Histological score of mice colonic tissue. Results are presented as mean ± SD, n = 7. ($^{\#\#}P < 0.01$ vs. NC; $^{*}P < 0.05$ vs. MC).

3.7. CJAF reconstructed damaged ultrastructure in IBS-D

To further investigate the effect of CJAF on the damaged colonic tissue of IBS-D mice, we utilized transmission electron microscope to observe the changes in ultrastructure of mice colonic tissue. As can be seen in NC group mice (Fig. 8A–F), the microvilli were arranged closely and neatly, the length was consistent, and the tight junction was complete and with dense staining. By contrast, for the MC group mice, the microvilli were sparsely distributed, and of different lengths, some even collapsed into small spots (as shown by yellow arrows). The tight junctions were lightly and unevenly stained (as shown by blue arrows). However, rifaximin and different dosages of CJAF treatment restored the ultrastructure damage to varying degrees.

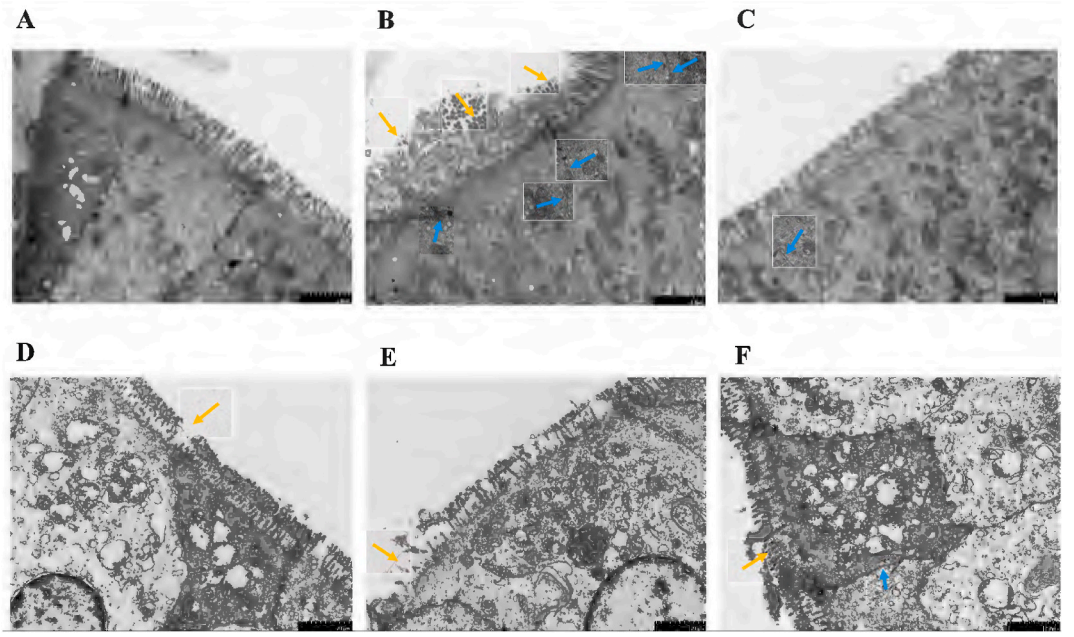

Fig. 8. CJAF restored ultrastructural damage of IBS-D mice. Representative electron micrographs of mice colonic tissues of (A) NC, (B) MC, (C) Rifaximin, (D) CJAF-H, (E) CJAF-M, and (F) CJAF-L. Yellow arrows indicate scattered and sparsely arranged microvilli, and blue arrows indicate light-staining and fractured junctional complex (scale bars 2 μm). All results are representative of at least three independent experiments.

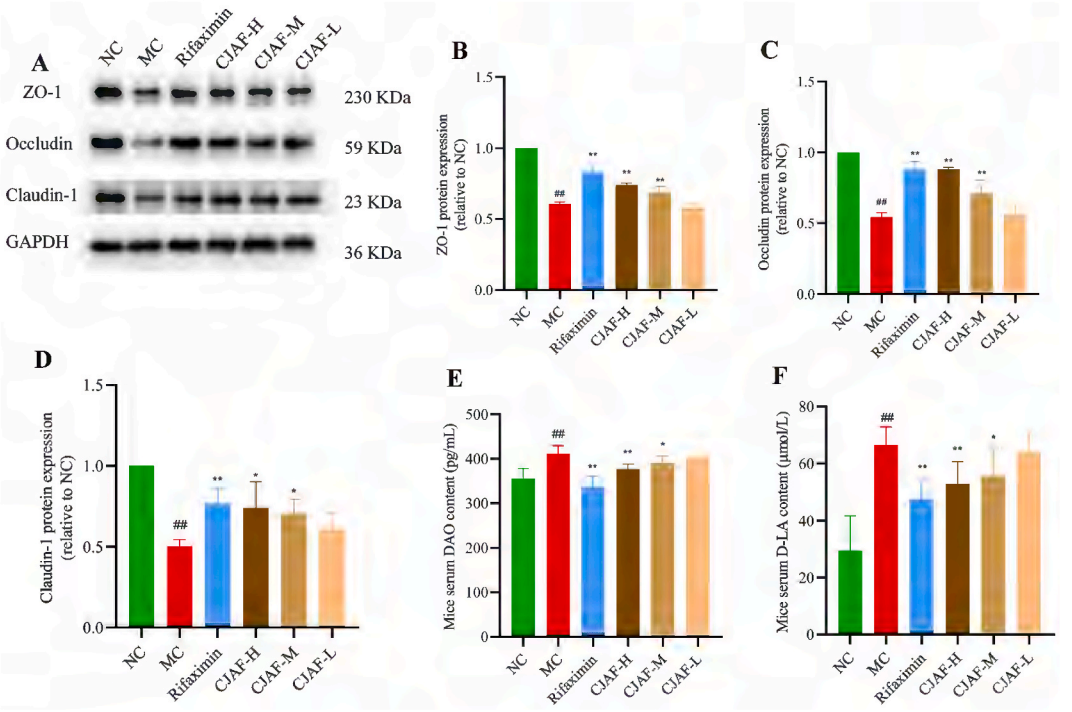

Fig. 9. CJAF restored intestinal barrier function in IBS-D mice. Representative Western blot images (A) and relative protein expression of ZO-1 (B), Occludin (C) and Claudin-1 (D) in mice colonic tissue. The full, non-adjusted images of Western blot was attached in supplementary material 3. Mice serum content of representative markers of intestinal epithelial integrity of DAO (E) and D-LA (F). Data are shown as mean ± the S.D. (B), (C), (D), n = 3; (E), (F), n = 7. $^{\#\#}P < 0.01$ vs. NC; $^{**}P < 0.01$, $^{*}P < 0.05$ vs. MC).

3.8. CJAF restored intestinal barrier function in IBS-D

Once activated, NLRP3 inflammasomes could promote pro-IL-1β and pro-IL-18 into matured IL-1β and IL-18 [34], which are potent pro-inflammatory cytokines. These pro-inflammatory cytokines could result in destruction of intestinal tight junction proteins and further lead to impaired intestinal barrier function [35]. Therefore, we investigated the expression of ZO-1, Occludin and Claudin-1 in mice colonic tissue. As depicted in Fig. 9A–D, IBS-D model construction decreased the expression of ZO-1, Occluding and Claudin-1($P < 0.01$) compared with that of NC group (Fig. 9A–D). However, after CJAF treatment, ZO-1, Occluding and Claudin-1 expression were remarkably increased as compared with the MC group ($P < 0.05$ or $P < 0.01$) (Fig. 9A–D). We further detected representative markers of intestinal epithelial integrity of DAO and D-LA in mice serum by ELISA. Compared with that of NC group, serum level of DAO and D-LA were upregulated in IBS-D model group ($P < 0.01$). Rifaximin, CJAF-H and CJAF-M treatment significantly downregulated DAO, D-LA levels ($P_{\text{rifaximin, CJAF-H}} < 0.01$, $P_{\text{CJAF-M}} < 0.05$, Fig. 9E–F), suggesting restoration of the integrity of intestinal epithelial barrier.

3.9. CJAF inhibited protein expression of NF-κB/NLRP3 pathway targets

The abnormal activation of the intestinal immune reaction plays a key role in the pathogenesis of IBS-D, associated with increased intestinal permeability [36]. The NLRP3 inflammasome plays a crucial part in innate immunity response by activating ASC and caspase-1 [34]. Previous studies showed that excessive activation of NLRP3 inflammasome pathway plays an important part in IBS-D pathophysiology associated with inflammatory response [37]. What's more, NF-κB is a key factor for the activation of NLRP3 inflammasome in the priming stage [38] and its binding to IκBα restraints the NF-κB: IκBα complex from transporting into the nucleus, thus keeping NF-κB in a quiescent condition [39]. Therefore, in the current study, we mainly focused on the role of NF-κB/NLRP3 pathway targets. The results obtained by Western blot showed that IBS-D model mice exhibited an increased protein expression of p-IκBα/IκBα, p-p65/p65, and NLRP3, ASC, pro-caspase-1, caspase-1 compared with that of NC group (Fig. 10A–H, $P < 0.05$ or $P <$

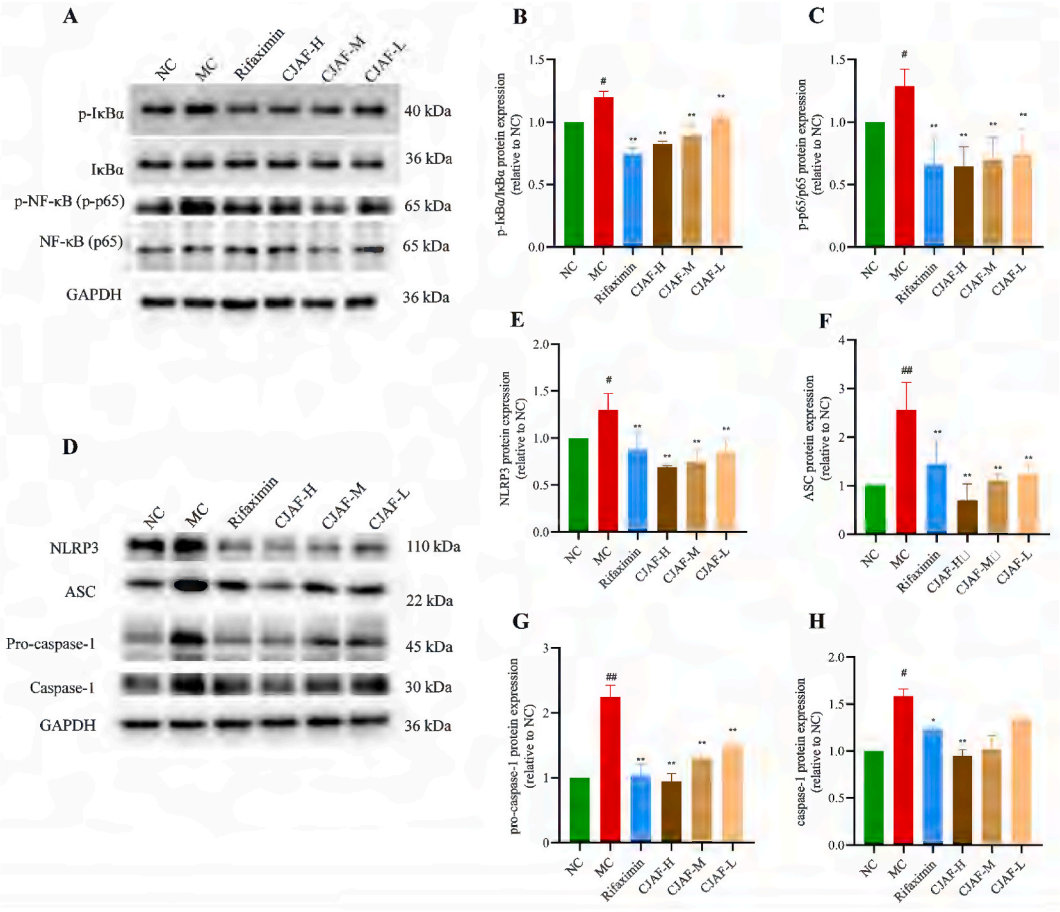

Fig. 10. CJAF inhibited protein expression of NF-κB/NLRP3 inflammasome pathway targets in IBS-D mice. Representative Western blot images of NF-κB/NLRP3 inflammasome pathway targets(A) (D). Relative protein expression of p-IκBα/IκBα(B), p-p65/p65(C), and NLRP3(E), ASC(F), pro-caspase-1(G), caspase-1(H). Data are shown as mean ± the S.D. n = 3. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. NC; $^{**}P < 0.01$, $^{*}P < 0.05$ vs. MC. The full, non-adjusted images of Western blot was attached in supplementary material 4.

0.01). Orally administration of rifaximin and high, moderate, low dosage of CJAF demonstrated dosage-dependently inhibitory effect on the protein expression of NLRP3 inflammasome pathway (Fig. 10A–H).

3.10. CJAF downregulated the proinflammatory cytokines in IBS-D

The activation of NLRP3 inflammasome lead to the release of proinflammatory cytokines, such as IL-1β and IL-18, which are associated with inflammatory response in the damage of intestinal mucous in IBS-D [40]. Therefore, we detected the protein expression of IL-1β, IL-18 and TNF-α in mice colonic tissue and further determined mice serum levels of IL-1β, IL-18, TNF-α and MPO by enzyme-linked immunosorbent assay. As depicted in Fig. 11A–D, IBS-D model induction significantly stimulated the protein expression of IL-1β, IL-18 and TNF-α in mice colonic tissue. However, after the intervention of CJAF, the protein expression of these proinflammatory cytokines were dose-dependently downregulated compared with that of MC group ($P < 0.05$ or $P < 0.01$). Additionally, the results for serum level of proinflammatory cytokines of TNF-α, IL-1β, IL-18, and MPO by ELISA were in accordance with that obtained in Western blot. As shown in Fig. 11E–H, after CJAF treatment, these proinflammatory cytokines were significantly downregulated ($P < 0.05$ or $P < 0.01$).

4. Discussion

In the current study, we employed a method of network pharmacology and found that the NF-κB signaling pathway, TNF signaling

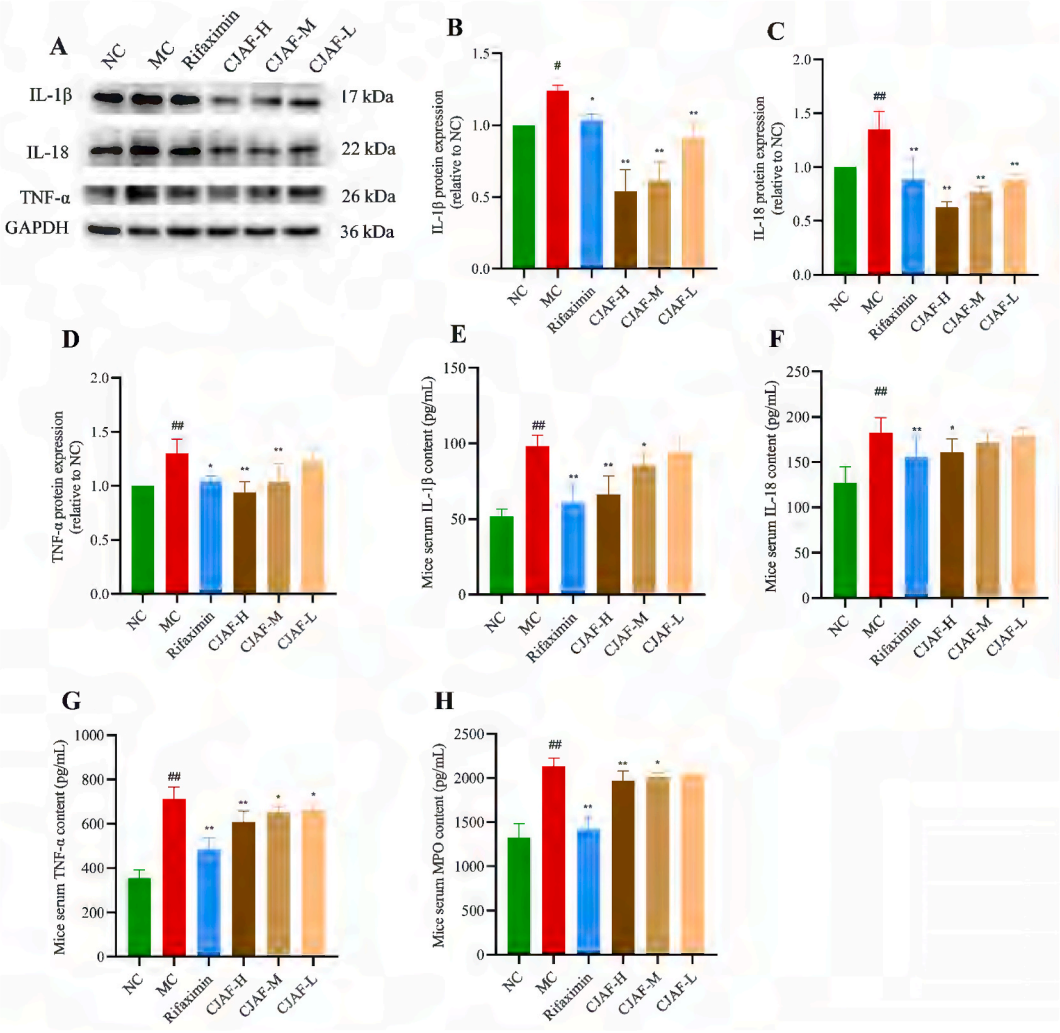

Fig. 11. CJAF downregulated the expression of proinflammatory cytokines in IBS-D mice. Representative Western blot images (A) and relative protein expression of IL-1β (B), IL-18 (C), TNF-α (D) in mice colonic tissue. The full, non-adjusted images of Western blot was attached in supplementary material 5. Mice serum content of proinflammatory mediators of IL-1β(E), IL-18(F), TNF-α(G) and MPO(H). Results are presented as mean ± the S.D. (B), (C), (D), n = 3; (E), (F), (G), (H), n = 7. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. NC; $^{**}P < 0.01$, $^{*}P < 0.05$ vs. MC.

pathway and IL-17 signaling pathway were intimately involved in the treatment of IBS-D for CJAF. These findings indicated that the therapeutic mechanisms of CJAF to treat IBS-D principally lies in the immune and inflammatory pathways. Further pharmacological tests on IBS-D mouse model confirmed that NF-κB/NLRP3 inflammasome pathway was extensively participated in the therapeutic mechanism of IBS-D by CJAF.

The 232 active ingredients optimized based on good ADME properties offered important information for further exploration on CJAF. Some of the active ingredients was reported to exert therapeutic effects against IBS-D. For example, atractylenolide I is the main active component and quality control component of *Atractylodes macrocephala Koidz.* in Chinese pharmacopoeia published in 2020. Atractylenolide I was shown to promote the restoration of intestinal epithelial through polyamine-mediated Ca^{2+} signaling pathway [41], which may be involved in therapeutic effect against IBS-D, for impaired intestinal epithelial barrier were proved to be an important pathological factor of IBS-D [42]. Paeoniflorin is the main bioactive ingredient of *Paeonia lactiflora Pall.*, which demonstrated analgesic effect on rat visceral hypersensitivity induced by neonatal maternal separation [43] and demonstrated anti-inflammatory effect by inhibiting NF-κB signaling pathway and eosinophil infiltration [44]. Astragaloside IV from *Astragalus membranaceus (Fisch.) Ege. var. mongholicus (Ege.) Hsiao* was proved to possess a therapeutic effect on colonic mucosal injury and accelerate epithelial cell proliferation to restore the mucosal barrier relevant proteins, possibly by attenuating the impaired ATP synthase [45]. Tetrahydropalmatine is an analgesic bioactive substance in *Corydalis yanhusuo W. T. Wang*, which exerted analgesic effects against inflammatory pain in rats by stimulating glial apoptosis as well as suppressing glial cells activation [46]. Berberine is an active constituent from *Coptis chinensis Franch.*, which is widely used to treat diarrhea in China for thousands of years. Researchers showed that berberine could alleviate visceral hypersensitivity in IBS-D model rats by restoring intestinal microecology and inhibiting the activation of spinal microglial [47]. Berberine was also shown to suppress intestinal inflammation and visceral hyperalgesia induced by stress, and reduce intestinal motility in IBS model rats [48]. Glycyrrhizin is the major bioactive chemical component from *Glycyrrhiza uralensis Fisch.*, which could ameliorate experimental colitis by inhibiting T cell responses and resultant decreased production of IL-17 [49]. What's unique for glycyrrhizin is that administration by rectal route obtained comparable protective effect against TNBS-induced colitis in rats, thus rectal administration may be a promising complementary treatment method [50,51]. Although differential diagnosis is required between colitis and IBS-D clinically, a majority of IBS-D mouse model was post-colitis, or more precisely, post-inflammation model, induced by chemical stimulus, such as TNBS or acetic acid [52]. Therefore, the above-mentioned pharmacological effect of ingredients from CJAF against intestinal inflammatory response could be extended to the mechanism of CJAF against IBS-D. Altogether, various components of CJAF may exert synergistic effect against IBS-D from aspects of inhibiting inflammatory response or analgesic effect against visceral pain.

Additionally, 56 crucial targets were identified as the candidate targets of CJAF for the treatment of IBS-D after PPI network analysis when the thresholds were set at DC $\geq$ 76. These kernel targets included TNF, IL6, ALB, AKT1, TP53, JUN, IL1B, VEGFA, STST3, CASP3, EGFR, MMP9, SRC, MAPK3, MYC, CXCL8, PTGS2, CCL2 and ESR1, etc. Our current work revealed that these genes were not only closely related to inflammatory response and immunity homeostasis in intestine, but also were intimately involved in intestinal motility, visceral nociception and in regulating gut-brain axis. For example, serum content of TNF-α were higher in IBS subtypes as compared with healthy controls and in women with IBS [53]. VEGF is one of the key targets in gastrointestinal mucosal remodeling and mucosal defense. VEGFA was proved to be closely related to chronic inflammation [54]. Previous study showed that ancient wheat products may relieve IBS symptoms by downregulating the expression of VEGFA [55]. IL-6 is a proinflammatory cytokine, which was shown to be significantly upregulated in patients with IBS-D as compared with that of healthy people [56]. Previous studies also showed gastrointestinal movement could be affected directly by IL-6 [57]. JUN, also known as c-Jun, is the most potent transcriptional activator of the AP-1 family [58]. Brain-derived neurotrophic factor (BDNF), a key role in gut-brain axis [59], was upregulated in the colonic mucosa of patients with IBS, and was regulated by IL-1β through a phosphorylated-c-Jun N-terminal kinase pathway [60].

According to network pharmacology analysis, the potential targets for CJAF against IBS-D were mainly associated with NF-κB, TNF and IL-17 signaling pathway. These signaling pathway were in strong link with inflammatory response and immune regulation. Our previous studies demonstrated that CJAF could attenuate symptoms of IBS-D in a rat model by regulating immune function and by improving intestinal permeability of IBS-D rats [11,12]. Combined with the results of network pharmacology analysis, we explored the effect of CJAF on NF-κB/NLRP3 signaling pathway in an IBS-D mice model.

NF-κB plays an important role in response to environmental changes and involved in many biological processes in organisms [39]. NF-κB connecting to IκB keeps the NF-κB: IκB complex from shifting to the nucleus, by which way retaining NF-κB in an inactive state. Once stimulated, IκB proteins will be phosphorylated and degraded, releasing NF-κB dimers. The freed NF-κB dimers could transfer into the nucleus and bind to specific sequences to promote or enhance the expression of target genes. What's more, NF-κB is a principal factor for the activation of NLRP3 inflammasome in the priming stage [38]. A previous study demonstrated that abnormal activation of NLRP3 inflammasome pathway could interrupt the enteric immune balance, the latter is closely related to IBS-D [37].

The results from the IBS-D mouse model experiments revealed that CJAF effectively relieved abdominal pain and diarrhea symptom of IBS-D as judged by AWR under CRD, diarrhea score and fecal water content in mice. Histological and ultrastructural observation demonstrated that treatment of CJAF ameliorated the tissue damage and inflammatory cell infiltration, restored microvilli and junctional complex integrity of mice colon tissue. Protein expression by Western blot for NF-κB/NLRP3 signaling pathway showed that CJAF could downregulate the protein expression of p-IκBα/IκBα, p-p65/p65, TNF-α, NLRP3, ASC, pro-caspase-1, caspase-1 and its downstream proinflammatory cytokines IL-1β, IL-18. This suggested that CJAF could inhibit inflammatory response and immune activation by inhibiting NF-κB/NLRP3 signaling pathway to treat IBS-D effectively. Previous studies showed that NF-κB is a key factor for priming NLRP3 inflammasome activation [38], which plays crucial part in the maintenance of intestinal homeostasis and modulates innate immune responses against commensal bacteria [40,61]. The NLRP3 inflammasome is an intra-cytoplasmic protein complex, which consists of NLRP3, ASC and pro-caspase-1 [62]. When stimulated, the inflammasome could transform the

pro-caspase-1 into active caspase-1, the latter then cleaves pro-IL-1β and pro-IL-18 to form matured IL-1β and IL-18 [63]. The release of IL-1β and IL-18 will finally initiate inflammatory response and immune activation in intestine, resulting in damage to intestinal epithelial tight junction and intestinal barrier function, which could be important in the pathogenesis of IBS-D [35,64,65]. Therefore, we also determined the expression of tight junction protein in mice colonic tissues. The results demonstrated that the regulating effect of CJAF on tight junction proteins of ZO-1, Occludin, and Claudin-1 displayed an opposite trend compared with the expression of proinflammatory cytokines IL-1β, IL-18, which showed a lower expression level in MC group and higher expression level in CJAF treatment group. Additionally, the results of ELISA of mice serum confirmed the trend in Western blot, in which the proinflammatory cytokines IL-1β, IL-18, TNF-α, MPO were downregulated after CJAF treatment. DAO, D-LA, reliable markers of intestinal epithelial integrity [66,67], were decreased in serum level after CJAF treatment, which could be the result of the restoration of intestinal epithelial barrier by upregulating ZO-1, Occludin, and Claudin-1. All these results indicated that CJAF could inhibit NF-κB/NLRP3 signaling pathway and downregulated the expression of proinflammatory cytokines IL-1β, IL-18, by which way the intestinal epithelial barrier was restored and the intestinal homeostasis was reconstructed.

Interestingly, rifaximin showed better repairing effect on tight junction protein than that of CJAF while demonstrated less effect on inhibiting NLRP3 inflammasome signaling pathway than CJAF as indicated by corresponding protein expression by Western blot experiment. This may be explained by that rifaximin is an antibiotic that is not absorbed by the gut, which kills or alters bacterial population in the gut [68], metabolites of the latter turned out to be a direct destructor of intestinal epithelial barrier [69]. Therefore, bacterial metabolites could be downregulated directly by rifaximin, resulting in a higher expression of tight junction protein expression of ZO-1, Occludin, and Claudin-1 by rifaximin than CJAF. However, CJAF, but not rifaximin, contains many chemical constituents that could potentially bind with NLRP3 inflammasome and inhibit the activation of this proinflammatory cascade as reported previously [25,70], leading to a relative more significant effect on NLRP3 signaling by CJAF (high and moderate, not low dose) than that of rifaximin.

Moreover, due to heterogeneity among patients with IBS, a universal treatment program is often difficult to achieve. A qualified management of IBS is based on personalized and comprehensive treatment strategies that include dietary, lifestyle, medical, and behavioral interventions [1]. More and more researches showed that the intestinal bacterial profile of patients with IBS differs from that of healthy subjects and enteric dysbacteriosis plays an important role in the pathogenesis of IBS [71,72]. Consequently, methods to repair intestinal microecological balance could be reasonably used to treat IBS. An efficient prospective treatment is fecal microbiota transplantation (FMT), which may be beneficial for recovery of bacterial diversity and improve enteric bacterial profile [73]. Although CJAF demonstrated effective against IBS-D in our previous studies [11,74,75], we are not sure whether the therapeutic mechanism was also related with its impact on intestinal microecology. This is also the weakness of this study, which is absent of analysis on the effects of CJAF on the intestinal flora of IBS-D mice, pointing the way for our future research. CJAF consists of 13 Chinese herbal drugs, chemical components of which was reported to exert anti-inflammatory [25], pain-relieving [46], antibacterial [47], and anti-depressant effect [76], thus the treatment of IBS by CJAF is the result of a comprehensive action of various effects. This could mean that CJAF may not be superior in a single term of antibacterial, anti-inflammatory or antidepressant effects, but it could do better in relieving general symptoms of IBS, such as abdominal pain and diarrheal, because of its multi-component and multi-target effect.

5. Conclusion

In conclusion, we utilized network pharmacology analysis to investigate potential mechanism of CJAF in the treatment of IBS-D and found that the molecular mechanisms mainly focused on the immune and inflammatory pathways, including NF-κB, TNF and IL-17 signaling pathway. Pharmacologic effects of CJAF against IBS-D were further evaluated in mice with TNBS enema combined with restraint stress-induced IBS-D model. And our experiment verified that CJAF inhibited NF-κB/NLRP3 signaling pathway activation, reduced inflammatory response, and alleviated visceral hypersensitivity and diarrhea symptom of IBS-D. Therefore, CJAF could be a therapeutic candidate for IBS-D therapy in the clinical practice.

Ethics statement

The animal experiment was reviewed and approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine (certificate no. 20220510014) and are in line with the ARRIVE guidelines.

Funding statement

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Data availability statement

Data will be made available on request. Active compounds of each Chinese medicine from CJAF and 219 potential targets of CJAF against IBS-D are included in excels in Supplementary information files. Original exposure maps of Western blot are included in PPT in Supplementary information files. The datasets of the current study are available in public database from TCMSP database (https://

tcmspw.com/tcmspsearch.php), PharmMapper (http://www.lilab-ecust.cn/pharmmapper/), GeneCards (http://www.genecards.org), Therapeutic Target Database (TTD, http://systemsdock.unit.osit.jp/iddp/home/inde), DrugBank (http://www.drugbank.ca/), DisGeNET (http://www.disgenet.org/), UniProt database (https://www.uniprot.org/), Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/), STRING database (https://string-db.org/), Omicshare Online tools (https://www.omicshare.com/tools), GO (https://geneontology.org/), and KEGG (https://www.kegg.jp/). More inquires of original data are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This research was approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine (certificate no. 20220510014). Animals were handled in accordance with the regulations of Guangzhou University of Chinese Medicine and in line with the ARRIVE guidelines.

Consent for publication

All authors agreed with the content of the manuscript and approved the final version of the manuscript.

CRediT authorship contribution statement

Wei Ke: Writing – original draft, Investigation, Data curation. **Jinjun Wu:** Software, Investigation. **Hongbin Li:** Investigation. **Siyu Huang:** Investigation. **Huibiao Li:** Investigation. **Yongfu Wang:** Investigation. **Yingxiu Wu:** Investigation. **Jie Yuan:** Investigation. **Shuncong Zhang:** Conceptualization. **Hongmei Tang:** Funding acquisition, Conceptualization. **Kaijun Lei:** Supervision, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

IBS-D,	irritable bowel syndrome with predominant diarrhea
TCM	traditional Chinese medicine
CJAF	Changji'an Formula
OB	oral bioavailability
DL,	drug likeness
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
TNBS	Trinitro-benzene-sulfonic acid
WAS	water avoidance stress
AWR	abdominal withdrawal reflex
CRD	colorectal distension
TEM	transmission electron microscopy
ELISA	Enzyme-linked immunosorbent assay
DAO	D-lactate and diamine oxidase
DLA	D-lactate
MPO	myeloperoxidase
TNF-α,	tumor necrosis factor
IL-1β	interlukine-1β
IL-18	interlukine-18
ZO-1	zonula occluden-1
IHC	immunohistochemistry

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33102.

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